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ACTIVITY-PREVENTING AND EGG-SEA-WATER NEUTRALIZING SUBSTANCES FROM SPERMATOZOA OF ECHINOMETRA SUBANGULARIS

WALTER E. SOUTHWICK

(*From the Bermuda Biological Station for Research* [1])

Introduction

A spermatozoön, like all highly specialized biological units, has many utterly unique, but very significant characteristics. Of these, probably the most fundamental is the finiteness of its period of life. As has been clearly demonstrated by Lillie (1919), Gray (1928) and others, mature spermatozoa, except possibly those of certain arthropods, are unlike all other biological units in that they do not at any time ingest or otherwise assimilate substances from which energy may be derived. Once, therefore, that a spermatozoön has been liberated from the testis, its every power and activity must be accomplished on the basis of substances that were located in its structure at the time of its liberation. Studies to determine what different substances might be located in a spermatozoön, the location, nature, and approximate amounts of such substances, the changes in location and state which they might undergo, and the factors which might influence, affect, or effect such processes are, therefore, especially important for an understanding of spermatozoan physiology. A series of studies which were made at the Bermuda Biological Station for Research have provided some observations concerning such substances.

Materials and Procedures

The studies were made with the common reef urchin, *Echinometra subangularis*, individuals of which were obtained from the reefs on the eastern end of St. David's Island. The trip to the reefs was made by bicycle, and the urchins were brought to the laboratory in a pail with a small volume of water and covered with seaweed. The water

[1] Laboratory space for this investigation was provided by the Bermuda Biological Station for Research, Inc. during July, 1938.

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was continually shaken, and so kept reasonably well aerated during the trip, a period of approximately twenty minutes.

Upon arrival at the laboratory, the urchins were immediately transferred to vessels in which a large volume of air was continually bubbled through a comparatively small volume of circulating sea-water. In such vessels, the urchins remained in good condition for approximately three days, and so trips were made regularly every two or three days in order to maintain a constant supply of freshly collected urchins.

The animals were opened by means of the usual circumferential cut, and the body fluids and intestines carefully removed. Every precaution was taken to avoid contamination, either of eggs with sperm, or of the sperm with egg secretions, and the gonads were removed from the test with glass needles. The ovaries were divided into small pieces in a small volume of sea-water with glass needles, and the mass strained through several thicknesses of unbleached cheesecloth. The supernatant fluid from the eggs was removed ten minutes later and fresh sea-water added. After two or three washings in this way, the supernatant fluid was allowed to stand for thirty minutes, at which time it would be capable of producing clear-cut and definite agglutinations with fresh sperm. The egg-sea-water was kept separate from the eggs during all subsequent tests. The testes were removed to a clean, dry Syracuse watch crystal, and, as fresh sperm was needed, the tubules were broken with a glass needle and the exuding dry sperm collected with a moderately fine pipette.[2] The watch crystal with the testes and the containers with the eggs or egg-secretions were kept covered at all times except when the materials were being withdrawn.

Movements of the Spermatozoa

When dry sperm is examined immediately under the microscope, the whole mass may be seen to be in a state of most intense vibratory activity. In such spermatozoa, however, due probably to the compactness of the mass, the active vibrations of the tails serve but to cause a rapid milling about of waving heads. Progressive movements are entirely absent, and the same group of spermatozoa sway back and forth, in constant, rapid vibration, but always in the same position, relative to each other and to the field of the microscope.

In such dry sperm, this motion lasts for from 50 to 120 seconds,

[2] Insemination tests made with such spermatozoa in a dilution proportion of one drop of dry sperm to 20 cc. sea-water, one drop of this suspension to one drop of eggs in 7 cc. sea-water gave, consistently, a fertilization percentage of from 92 to 100 per cent.

and then all movement ceases. The cessation in activity, once it has started, spreads rapidly, so that the entire transition from universal activity to complete quiescence is accomplished within about 15 seconds. Such spermatozoa do not show motion when they are redistributed mechanically by means of pressures on the cover glass.

When, however, a drop of sea-water is brought in contact with a drop of dry sperm, the edge of the drop of dry sperm "frays" slightly. After about one minute, the spermatozoa in this frayed edge begin to move slowly. Gradually, they come to move more rapidly, more spermatozoa become active, and they move slowly out into the drop of sea-water. In about two minutes, the whole mass, or as much of it as is reached by the diffusing sea-water, becomes intensely active. In the denser portions of this mass, as in the original drop of dry sperm, the motion is essentially a rapid milling about of waving heads with progressive movements entirely absent. In the less dense portions, however, progressive movements do occur.

When the dilution of one drop of dry sperm to one drop of sea-water is made upon a glass slide where such changes in the supporting medium as might be produced by evaporation are minimized by means of placing pure petroleum jelly around the edges of the cover glass, the activity continues, with gradually diminishing intensity, for about three or four hours, when all motion ceases. If another drop of sea-water then be added to the suspension under the cover glass, the spermatozoa again become active, and this activity continues with gradually decreasing intensity for about one and one-half hours, when all motion again ceases. Such spermatozoa can be reactivated again by the addition of another drop or two of sea-water, but the length of time during which the spermatozoa remain active with each successive reactivation progressively decreases until finally, after about eight such reactivations, no further activation is obtained. This phenomenon is shown more clearly in Table I which gives the actual observations of a typical experiment of this nature.

If the reactivation obtained from the addition of fresh sea-water to a suspension of inactivated spermatozoa were due to dilution effects, then one might expect a substance to occur in the suspensory fluid of an inactivated suspension of spermatozoa which would have the property of preventing dry sperm from becoming active. In order to test this possibility, a suspension of dry sperm was centrifuged for 30 minutes at 4,000 revolutions per minute. When this centrifuging had been completed, a clear, slightly opalescent, bluish fluid comprising about 11 per cent of the total volume of the original dry sperm had separated from the mass of spermatozoa. Utmost care had been

used to have the centrifuge tubes clean and *perfectly dry* before the spermatozoa were added, and great care was now used to have pipettes, glass slides, and cover glasses all perfectly dry. A drop of the clear supernatant fluid was removed from the centrifuge tube and carefully

TABLE I

Changes in the activity of a suspension of spermatozoa with time, and the effects of repeated additions of fresh sea-water upon the duration and changes in the activity of the spermatozoa in such suspensions.

Time	Observations on the Activity	Treatment of the Suspension	Number of the Sea-water Additions	Time Interval between Sea-water Additions
10:45		1 dr. dry sperm	0	
10:45	Intensely active			
10:47	All sperm utterly motionless	1 dr. sea-water	1	
10:48	Intensely active			
11:02	Active			
11:13	Some motion			
11:29	Some motion			
11:44	A little motion			
12:05	A little motion			
12:26	A little motion			
1:21	Still a little motion			
1:48	Just a little motion			
2:12	No motion	1 dr. sea-water	2	3 hours 27 min.
2:13	Intensely active			
2:39	Active			
2:49	Active			
3:27	Some activity			
3:53	Motionless	Sea-water added	3	1 hour 40 min.
3:57	Activation			
4:50	Motionless	Sea-water added	4	57 min.
4:52	Some activity			
5:12	Active			
5:51	Motionless	Sea-water added	5	61 min.
5:55	Some activity			
6:25	Active			
7:03	Motionless	Sea-water added	6	72 min.
7:05	Some activity			
7:47	Motionless	Sea-water added	7	44 min.
7:51	A little activity			
8:22	Motionless	Sea-water added	8	35 min.
8:30	No further activation			

brought in contact with a drop of dry sperm. The whole process of fusion of the two drops was carefully watched with the microscope, but there was no slightest sign of any activation whatsoever at any time. Even when the spermatozoa were thoroughly distributed throughout the supernatant sperm fluid by means of stirring with a

clean dry glass needle, there was no activation, and even examinations with a magnification of 950 × failed to show any activity whatsoever. Dilution of dry spermatozoa from the same testis tubules with ordinary sea-water gave perfectly typical activation.

Two preparations with the supernatant fluid were made permanent with the edges of the cover glass sealed with petroleum jelly, one at 12:35 P.M., and one at 1:55 P.M. The spermatozoa in the former were still motionless at 2:21 P.M. but when fresh sea-water was added at that time, the spermatozoa became intensely active by 2:25 P.M. In the latter, the spermatozoa remained motionless until 7:30 P.M., but when fresh sea-water was added at that time, became intensely active by 7:37 P.M. *The residual spermatozoa after centrifuging showed normal activation in all cases when ordinary sea-water was added.*

This experiment definitely indicated that the suspensory fluid of dry spermatozoa presents a condition which serves to prevent fresh dry sperm from becoming active. This condition occurs also, in the supernatant fluid, when 3″ of dry sperm is centrifuged for 30 minutes at 2,500 revolutions per minute through 1½″ of fresh sea-water. If the supernatant fluid from such centrifuging be removed and replaced with fresh sea-water, and the spermatozoa of the first centrifuging be centrifuged through this sea-water for 45 minutes at 2,500 revolutions per minute, the supernatant fluid from this centrifuging, too, will prevent the activation of dry sperm.

All gradations occur. All supernatant fluid obtained by any system of centrifuging dry sperm will prevent activation of fresh sperm. When more dry sperm than sea-water is present, one passage of the spermatozoa through the sea-water is sufficient to render the supernatant fluid capable of preventing activation of dry sperm. When, however, more sea-water than dry sperm is present, several washings, the number depending upon the condition of the spermatozoa and the relative proportions of sperm and sea-water, are necessary, and there are several experiments which demonstrate the relationship between the relative amount of dry sperm and sea-water present to the amount of centrifuging necessary to cause the supernatant fluid to prevent activity in dry sperm. Thus, in one typical experiment, dry sperm to sea-water in the proportions of ⅜″ : 3¼″, 1$\frac{3}{16}$″ : 2¾″, 2⅜″ : 2½″, and 1⅞″ : 1½″ were each washed five times, being centrifuged approximately 10 minutes at 3,550 revolutions per minute for each washing. The supernatant fluid from the ⅜″ : 3¼″ proportion did not stop dry sperm, whereas the supernatant fluid from each of the other proportions stopped dry sperm completely. In another typical experiment, the proportion of dry sperm to sea-water was made the same

in all four tubes, namely ½″ : 3″, and a tube removed after 5, 10, 15, and 20 washings of approximately four minutes at 3,250 revolutions per minute for each washing. The supernatant fluid after five washings did not stop dry sperm, the fluid obtained after 10 washings allowed "just a little activity" in dry sperm, while the supernatant fluid obtained after 15 and 20 washings completely prevented activity in dry sperm.

In order to make certain that the occurrence of the condition in the supernatant fluid was developed by the centrifuging, an exactly similar proportion of dry sperm and sea-water was made and thoroughly mixed at the same time that the other tubes were prepared. This suspension of spermatozoa was kept in a tube until the centrifuging of the other tubes had been completed. The sperm in this control tube were then separated from the suspensory fluid by means of one centrifuging at 3,250 revolutions per minute for 8 minutes, and the resulting supernatant fluid tested. It was found to be utterly incapable of preventing or reducing activity in dry sperm to any extent that could be optically determined.

These experiments indicate that some substance or condition develops or appears in suspensions of spermatozoa which has the property of preventing activity in the spermatozoa of that or of a fresh suspension of dry sperm. This substance or condition is rendered ineffective, or is reduced to sub-threshold concentration or intensity by the addition of fresh sea-water, but its concentration or intensity may be definitely and markedly increased by washing the spermatozoa through the suspensory fluid by means of the centrifuge.

Other Properties of Supernatant Sperm Fluid

When a drop of supernatant fluid which will definitely prevent activity in dry sperm is thoroughly mixed, by means of a clean, dry glass needle, with one drop of an egg-sea-water which will produce definite and clear-cut agglutination clumps with dry sperm and this mixture, in the form of a drop on a clean, dry glass slide, is brought into contact with a drop of dry sperm from the same tubules, and the whole process watched carefully with the microscope, the spermatozoa will be seen to become active in a manner exactly similar to that which occurs when a dilution is made with ordinary sea-water, but no slightest sign of any form or degree of agglutination whatsoever occurs. This test was repeated several times with many different preparations, and with many different lots of gametes, but always with the same result. In all cases when the supernatant sperm fluid would prevent activity in dry sperm, it would neutralize or destroy in some way the ag-

glutinating power of egg-sea-water, but reciprocally, its power to prevent activity in dry spermatozoa was similarly destroyed, and the mixture of supernatant fluid plus egg-sea-water served to activate spermatozoa in a manner exactly similar to that of ordinary sea-water.

This phenomenon was even more vividly shown when a small drop of the supernatant sperm fluid was added, with a fine capillary pipette, to the middle of the field where the agglutinated clumps, produced by the addition of egg-sea-water to dry sperm, could everywhere be seen, and the whole process watched continuously under the microscope. The response was almost instantaneous. The agglutination clumps everywhere within the area of the added drop *immediately* dispersed, while outside the boundary of the drop and on all sides, clumps persisted in a perfectly typical and unaffected arrangement. Within the drop, the spermatozoa instantly became inactive, while along the boundary of the drop, some activity and motion could be seen.

The phenomenon, too, was readily demonstrated by making preparations on one slide which could be observed successively and sequentially, and directly compared, and which would consist of dry sperm mixed with (1) ordinary sea-water, (2) egg-sea-water, (3) supernatant sperm fluid, and (4) one drop egg-sea-water plus one drop supernatant sperm fluid. (1) and (4) showed typical activation, but no agglutination, (2) showed distinct and definite agglutination, while the spermatozoa in (3) were utterly inactive.

There seems to be a parallelism between ability to prevent activity of dry sperm and ability to neutralize egg-sea-water. All supernatant fluid that would prevent activity in dry sperm would also neutralize the agglutinating power of egg-sea-water. When the supernatant fluid allowed "just a little activity," the mixture of supernatant sperm fluid and egg-sea-water allowed "slight and evanescent tendencies" towards the formation of agglutination clumps, while finally, the supernatant fluid obtained from a sperm suspension that had been diluted for 2¾ hours, but that had not been washed by centrifuging, would not prevent activity of dry sperm, and would not prevent egg-sea-water from causing agglutination. These observations seem to indicate that the activity-preventing and the egg-sea-water neutralizing properties of supernatant sperm fluid are due, either to the same substance or condition, or to two or more substances which, however, arise or occur together in the supernatant fluid under all the treatments where the effects have been observed.

Discussion

Attempts to extract such substances from the spermatozoön as would have a relationship to the fertilization reaction have been

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made by Sampson (1926), Hibbard (1928), Wintrebert (1929, 1930*a*, 1933), Einsele (1930), and Parat (1933*a*). Sampson was unable to obtain any substances in sperm filtrates and dialysates which would activate "fertilizin" in the egg-sea-water so as to make it an efficient parthenogenetic agent, or would combine with the agglutinating substance in egg-sea-water so as to destroy its power to agglutinate fresh sperm suspensions, though she did obtain substances which would initiate development of mature ova of the same species. Hibbard and Wintrebert found that solutions of macerated spermatozoa would digest egg membranes, while Einsele and Parat found that filtered ether dialyzates and extracts of entire testes would, when injected into the egg with a micropipette, give an activation of the eggs in 60 per cent of the cases. Parat found that the development was much more regular, and that many more of the gastrulae would form larvae when the eggs were activated parthenogenetically by means of the introduction of the acrosome of spermatozoa removed and injected by means of microdissection needles and pipettes. Both Parat and Einsele have obtained evidence that the substance concerned in such parthenogenetic activations is a proteolytic enzyme.[3] Histo-anatomical studies of spermatozoa have been made by Bowen (1924), Popa (1927), Wintrebert (1930*b*), Parat (1928, 1933*b*) and others, and some of these studies indicate the presence of substances of secretory origin in the acrosomal region of spermatozoa.

Since egg-sea-water, reciprocally, will neutralize or destroy the activity-preventing or inhibiting substance of a sperm suspension, the activating property of egg-sea-water, as described by Lillie (1913), hereby receives an explanation. The power of the substances in the supernatant sperm fluid to neutralize the agglutinating power of egg-sea-water also conforms very readily with the fertilizin theory of Lillie (1919), and with the observations of Lillie (1919) and others with *Arbacia punctulata* that it was not possible at any time to regain agglutinating substances that had once been used in order to cause an agglutination of spermatozoa.

Summary

1. Dry sperm of *Echinometra subangularis* is intensely active immediately after its removal from the testes tubules, but this motion

[3] Recently (1939), Frank has described a sperm extract of *Arbacia punctulata* spermatozoa, obtained by heating the sperm suspension, which has the property of neutralizing the fertilizin of egg-sea-water, and which has an agglutinating effect on the cilia of *Arbacia* plutei. It is possible that the inactivating effect of the supernatant fluid of *Echinometra* spermatozoa is due to a similar action upon the tails of the sperm.

lasts for but from 50 to 120 seconds, when all activity ceases. When, however, a drop of sea-water is brought in contact with such a drop of dry sperm, the spermatozoa again become active, and this activity continues for from three to four hours. The addition of another drop of sea-water will cause the spermatozoa again to become active, and this activity continues with gradually decreasing intensity for about one and one-half hours. Such spermatozoa can again be reactivated, but the length of time during which they remain active progressively decreases, until finally, no further activation is obtained.

2. This inactivation of sperm suspensions with time is caused, probably, by the accumulation of a substance with time which has, as its characteristic identificatory property, the prevention of spermatozoa from activity. It occurs in the supernatant fluid obtained from centrifuging dry sperm, and in the supernatant fluid obtained by washing dry sperm through ordinary sea-water several times with the centrifuge, but the suspensory fluid of diluted sperm suspensions through which the spermatozoa have not been washed does not contain the substance in detectable amounts.

3. Supernatant fluid which contains this substance also, in all cases, has the property of neutralizing the agglutinating power of egg-sea-water, and there seems to be a parallelism between ability to prevent activity of dry sperm and ability to neutralize egg-sea-water.

4. These observations indicate that the activity-preventing and the egg-sea-water neutralizing properties of supernatant fluid are due, either to the same substance, or to two substances which, however, occur together under all the treatments where the effects have been observed.

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